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FAX NUMBER: 571-273-8300
571-273-1085

CLIENT MATTER #: 04393/0202300-US0

OF PAGES (including cover): 16

FROM:
Shilpa V. Patel, Ph.D.
Attorney at Law
212.527.7630
spatel@darbylaw.com

COMMENTS:

NEW YORK
7 WORLD TRADE CENTER
250 GREENWICH STREET
NEW YORK, NY 10007-0042
TEL 212 527.7700
FAX 212.527.7701
www.darbylaw.com

NEW YORK
SEATTLE
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SAN JOSE
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ATTORNEY AT LAW
212.527.7630
spatel@darbylaw.com

VIA FACSIMILE (571-273-8300; 571-273-1085)

Maria G. Leavitt, Ph.D.
Group Art Unit 1633
United States Patent & Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

Re: **U.S. Patent Application Serial No.: 10/540,302**
For: THE METHOD OF INDUCING HOMOLOGOUS RECOMBINATION OF SOMATIC CELL

Dear Examiner Leavitt:

Further to our conversation of today, we enclose copies of (Van Gent (1996) and Arakawa and Buerstedde (2004) that we will refer to tomorrow and we will subsequently file in an Information Disclosure Statement.

Additionally, tomorrow we will refer to Bassing (2000) and Agata et al. (2001), both of which previously have been made of record.

Please confirm by return facsimile your receipt of these documents.

Sincerely yours,

Shilpa V. Patel, Ph.D.

SVP:ss
Enclosures

Cell, Vol. 85, 107–113, April 5, 1996, Copyright ©1996 by Cell Press

The RAG1 and RAG2 Proteins Establish the 12/23 Rule in V(D)J Recombination

Dik C. van Gent, Dale A. Ramsden, and Martin Gellert
Laboratory of Molecular Biology
National Institute of Diabetes and Digestive and Kidney Diseases
National Institutes of Health
Bethesda, Maryland 20892

Summary

V(D)J recombination requires a pair of signal sequences with spacer lengths of 12 and 23 base pairs. Cleavage by the RAG1 and RAG2 proteins was previously shown to demand only a single signal sequence. Here, we establish conditions where 12- and 23-spacer signal sequences are both necessary for cleavage. Coupled cutting at both sites requires only the RAG1 and RAG2 proteins, but depends on the metal ion. In Mn^{2+}, a single signal sequence supports efficient double strand cleavage, but cutting in Mg^{2+} requires two signal sequences and is best with the canonical 12/23 pair. Thus, the RAG proteins determine both aspects of the specificity of V(D)J recombination, the recognition of a single signal sequence and the correct 12/23 coupling in a pair of signals.

Introduction

In lymphoid cells, mature immunoglobulin and T-cell receptor genes are assembled from separate gene segments by V(D)J recombination (Gellert, 1992; Lewis, 1994). This process is directed by recombination signal sequences (RSSs), which flank the coding segments. An RSS is made up of conserved heptamer and nonamer motifs, separated by a spacer with nonconserved sequence but a relatively conserved length of 12 or 23 base pairs (bp).

V(D)J recombination can be divided into two stages. First, double-strand breaks (DSBs) are made at the coding/signal borders. Such DSBs have been detected at T-cell-receptor (Roth et al., 1992a) and immunoglobulin loci (Schlissel et al., 1993). Later, pairs of coding ends and signal ends are joined. Signal ends have been found in all rearranging cells and shown to be intermediates leading to signal joints (Ramsden and Gellert, 1995). Coding ends were initially only detected in mice carrying the severe combined immunodeficiency (*scid*) mutation (Roth et al., 1992b), but have also recently been found in a non-*scid* background (Ramsden and Gellert, 1995).

DSBs are now known to be made by the RAG1 and RAG2 proteins in a two-step reaction (McBlane et al., 1995; van Gent et al., 1995). First, a nick is introduced at the 5′ end of the RSS heptamer, leaving a 3′-OH on the coding side, and a 5′ phosphate on the signal side. This 3′-OH is then used to attack the phosphodiester bond in the other strand opposite the initial nick by direct transesterification (van Gent et al., 1996), resulting in a hairpin coding end and a blunt, 5′ phosphorylated signal end.

After DSB formation, the hairpin coding ends are opened by an as yet unknown mechanism and coupled to form a coding joint, and the signal ends are joined in a head-to-head fashion (signal joint). The joining reactions require several factors that are also involved in general DSB repair (Jackson and Jeggo, 1995).

We have shown previously that the RAG1 and RAG2 proteins are able to cleave oligonucleotide substrates containing a single RSS, resulting in the formation of a hairpin coding end and a blunt, 5′ phosphorylated signal end. In vivo, recombination takes place between one RSS with a 12 bp spacer (12-signal) and one with a 23 bp spacer (23-signal); this is the so-called 12/23 rule. DSB formation in vivo also depends on the presence of such a pair of RSSs (S. R. Steen, L. Gomelsky and D. B. Roth, personal communication), indicating that the 12/23 rule is linked to the initial cleavage event. However, with Mn^{2+} as divalent cation, cleavage by the purified RAG1 and RAG2 proteins did not demand a second RSS, nor did the presence of a partner signal stimulate this reaction. Here we show that with Mg^{2+} as divalent cation, efficient cleavage requires the presence of a 12-signal and a 23-signal but does not occur at a single RSS, thus recapturing the 12/23 rule in vitro with only the RAG1 and RAG2 proteins.

Results

Effect of Divalent Cation on Cleavage

As shown before, a single RSS is cleaved by the purified RAG1 and RAG2 proteins (McBlane et al., 1995). The two products, the nicked species resulting from the first step and the hairpin resulting from the second, can be seen in Figure 1 (lane 2). Efficient cleavage of such an oligonucleotide substrate requires Mn^{2+} as divalent cation; in the presence of Mg^{2+}, only the nicked species is made (Figure 1, lane 3).

In the presence of Mn^{2+}, DNA substrates containing two RSSs (one with a 12 bp spacer and one with a 23 bp spacer) were found to be cleaved at either RSS, independent of the other (McBlane et al., 1995; van Gent et al., 1995). We investigated whether substitution of Mg^{2+} for Mn^{2+} might restore the need for a second RSS. To allow the two RSSs to be aligned without any hindrance by limited DNA flexibility, we inserted a 0.9 kb fragment between the signals (pDVG42; see Figure 2A). This plasmid was linearized with the restriction enzyme AatII and incubated with RAG1 and RAG2 in the presence of either Mn^{2+} or Mg^{2+}. Reaction products were analyzed by Southern blotting, using the 0.9 kb insert as probe. Cleavage at both signals generates a 1 kb product; cleavage at only the 12-signal or only the 23-signal will yield 6 kb or 3 kb products, respectively. In the presence of Mn^{2+}, high levels of the 6 kb and 3 kb products were observed (29% and 9% of total substrate, respectively), showing that cuts were made efficiently at one RSS without cleavage at the other (Figure 2B, lane 2). The 1 kb product, arising from cleavage at both signals, was also observed (7%), but at a level that was not significantly higher than expected for two independent cleavage events.

In Mg^{2+}, however, the majority of products was cut at both signals (13%), with only a small minority cleaved just at the 12-signal (3%) and barely detectable single

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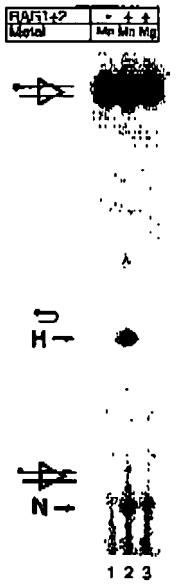

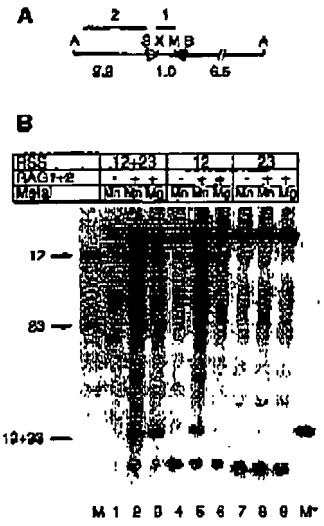

Figure 2. Coupled Cleavage in Mg^{2+}

(A) Schematic representation of recombination substrate pDVG42. The RSSs are depicted by triangles (open for the 12-signal and